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## TITLE

Transferrin-bearing polypropylenimine dendrimer for targeted gene delivery to the brain

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## ABSTRACT

The possibility of using genes as medicines to treat brain diseases is currently limited by the lack of safe and efficacious delivery systems able to cross the blood-brain barrier, thus resulting in a failure to reach the brain after intravenous administration.

On the basis that iron can effectively reach the brain by using transferrin receptors for crossing the blood-brain barrier, we propose to investigate if a transferrin-bearing generation 3- polypropylenimine dendrimer would allow the transport of plasmid DNA to the brain after intravenous administration.

*In vitro*, the conjugation of transferrin to the polypropylenimine dendrimer increased the DNA uptake by bEnd.3 murine brain endothelioma cells overexpressing transferrin receptors, by about 1.4-fold and 2.3-fold compared to that observed with the non-targeted dendriplex and naked DNA. This DNA uptake appeared to be optimal following 2h incubation with the treatment.

*In vivo*, the intravenous injection of transferrin-bearing dendriplex more than doubled the gene expression in the brain compared to the unmodified dendriplex, while decreasing the non-specific gene expression in the lung. Gene expression was at least 3-fold higher in the brain than in any tested peripheral organs and was at its highest 24h following the injection of the treatments.

These results suggest that transferrin-bearing polypropylenimine dendrimer is a highly promising gene delivery system to the brain.

## KEYWORDS

Brain delivery; blood-brain barrier; gene delivery; dendrimer; transferrin

## 1. Introduction

Gene therapy has emerged as a promising strategy to treat cerebral diseases such as glioma, Alzheimer's and Parkinson's diseases, which affect a large percentage of the world's population and hardly respond to intravenously administered, small molecule treatment [1-4]. Although the genetic basis for many of these diseases is known, the possibility of using genes as medicines is currently limited by the lack of safe and efficacious delivery systems able to cross the blood-brain barrier (BBB) and to deliver DNA to the brain after intravenous administration.

The BBB acts as an entrance gateway, restricting the movement of ions and nutrients to the central nervous system while protecting the brain against harmful blood-borne substances and invading organisms [2, 5]. Its permeability properties prevent the delivery of more than 98% of drugs, including nucleic acids, to the brain [2, 3]. In addition, locally administered treatments fail to achieve a widespread gene expression in the target cells throughout the entire brain, which is necessary for a successful treatment of most cerebral pathologies [2, 3, 6].

However, the BBB does possess specific receptor-mediated transport mechanisms that can potentially be exploited as a means to target drugs and genes to the brain. The transferrin receptor (TfR) is of particular interest because it is overexpressed on the brain capillary endothelial cells [7]. The antibodies that bind to the TfR have been shown to selectively target the brain microvascular endothelium due to the high levels of TfR expressed by these cells [8-10]. This strategy has been widely investigated for the delivery of drugs and genes to the brain [11].

Several strategies have been explored to formulate TfR-targeted delivery systems able to transport nucleic acids to the brain following intravenous administration [11]. Numerous non-viral gene delivery systems are currently under development, due to their low immunogenicity, stability, unrestricted plasmid size and versatility in types of modifications [12, 13]. Among these delivery systems, generation 3- diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. We recently prepared a transferrin (Tf) -bearing generation 3- diaminobutyric polypropylenimine dendrimer (DAB-Tf), able to increase the cellular uptake and gene expression of DNA by cancer cells overexpressing transferrin receptors compared to non-targeted delivery systems, *in vitro* and *in vivo* [14]. Importantly, the treatment was well tolerated by the animals, with no apparent signs of toxicity.

Building on this study, we now would like to investigate if this Tf-bearing gene delivery system could improve the delivery of DNA to the brain, *in vitro* and *in vivo* following intravenous administration.

## 2. Materials and methods

### 2.1. Cell lines and reagents

Human holo-transferrin, generation 3- diaminobutyric polypropylenimine dendrimer (DAB), dimethylsuberimidate and all other chemicals and reagents that are not specifically mentioned below were obtained from Sigma Aldrich (Poole, UK). The expression plasmids encoding  $\beta$ -galactosidase (pCMVSPORT  $\beta$ -galactosidase) and tdTomato (pCMV-tdTomato) were respectively purchased from Invitrogen (Paisley, UK) and Clontech (Mountain View, CA). They were propagated in *E. coli* and purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Vectashield<sup>®</sup> mounting medium with 4',6-diamidino-2-phenylindole (DAPI) came from Vector Laboratories (Peterborough, UK). Passive lysis buffer, Label IT<sup>®</sup> Cy3- and fluorescein- Nucleic Acid Labeling kits were respectively obtained from Promega (Southampton, UK) and Cambridge Biosciences (Cambridge, UK). bEnd.3 murine brain capillary endothelial cell line was purchased from LGC Standards (Teddington, UK), while cell culture media were obtained from Invitrogen (Paisley, UK).

## *2.2. Synthesis and characterization of transferrin- bearing DAB dendrimer*

Transferrin (Tf) was conjugated to generation 3- diaminobutyric polypropylenimine dendrimer (DAB) by using dimethylsuberimidate (DMSI) as a cross-linking agent, as previously reported [14, 15]. DAB (24 mg) was added to transferrin (6 mg) and dimethylsuberimidate (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The reaction took place for 2 h at 25 °C whilst stirring. The conjugate was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The conjugation of Tf to DAB was assessed by <sup>1</sup>H NMR spectroscopy using an Oxford NMR AS 400 spectrometer (Jeol, Peabody, MA).

## *2.3. In vitro biological characterization*

### *2.3.1. Cell culture*

Immortalized bEnd.3 cells overexpressing Tf receptors were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured at 37°C in a humid atmosphere of 5% carbon dioxide.

### *2.3.2. Cellular uptake*

Imaging of the cellular uptake of the DNA carried by DAB-Tf was carried out using epifluorescence microscopy. Labeling of the  $\beta$ -galactosidase-encoding plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT<sup>®</sup> Cy3 Nucleic Acid Labeling kit, as described by the manufacturer. bEnd.3 cells were seeded on coverslips in 6-well plates ( $10^4$  cells/ well) and grown at 37°C for 72 h. They were then incubated for different durations (15, 30, 45, 60, 120, 240 min) with Cy3-labeled DNA (2.5  $\mu$ g DNA / well) complexed to DAB-Tf at the dendrimer: DNA weight ratio of 10:1. The cells were then washed three times with PBS and fixed with methanol for 10 min. Upon staining of the nuclei with DAPI, the cells were examined using an E600FN Upright Epifluorescence microscope (Nikon, Tokyo, Japan). DAPI was excited with the 405 nm laser line (bandwidth: 415-491nm), whereas Cy3 was excited with the 543 nm laser line (bandwidth: 550-620 nm).

Once the treatment duration allowing maximal DNA uptake was determined, a similar procedure was performed to compare the cellular uptake of Cy3-labeled DNA (2.5  $\mu$ g/well) complexed to DAB-Tf and DAB (dendrimer: DNA weight ratios respectively of 10:1 and 5:1 [14, 16] during the optimized treatment duration. Control samples were treated with naked DNA or remained untreated.

Quantification of cellular uptake was performed using flow cytometry. Labeling of plasmid DNA with the fluorescent probe fluorescein was performed using a Label IT<sup>®</sup> Fluorescein Nucleic Acid Labeling kit, as described by the manufacturer. bEnd3 cells were grown in 6-well plates ( $1.6 \times 10^5$  cells / well) at 37 °C for 72 h. The cells were then treated with fluorescein-labeled DNA (5  $\mu$ g DNA / well), alone or complexed to DAB-Tf and DAB (dendrimer: DNA weight ratios respectively of 10:1 and 5:1). Untreated cells served as a negative control. After 2 h incubation with the treatments, single cell suspensions were prepared, washed (2 mL PBS pH 7.4 per well) and pelleted (378 g for 8 min) 3 times, before being analyzed using a FACSCanto<sup>®</sup> flow cytometer (BD, Franklin Lakes, NJ). Ten thousand cells (gated events) were counted for each sample. Their mean fluorescence intensity was analyzed with FACSDiva<sup>®</sup> software (BD, Franklin Lakes, NJ).

### *2.3.3. Mechanisms of cellular uptake of DNA complexed to DAB-Tf dendriplex*

The mechanisms involved in the cellular uptake of DNA complexed to DAB-Tf dendriplex were investigated by treatment with uptake inhibitors and escalating concentrations of free Tf. Cells were seeded and grown as described above. After removal of the medium, they were then pre-treated with phenylarsine oxide (10  $\mu$ mol/L), filipin (5  $\mu$ g/mL), colchicine (10  $\mu$ mol/L), poly-L-lysine (400  $\mu$ g/mL) and various concentrations of free Tf ranging from 2.5

to 20  $\mu\text{mol/L}$  for 10 min at 37°C. The cells were then treated with fluorescein-labeled DNA (respectively 2.5 and 5  $\mu\text{g/well}$  for qualitative and quantitative analysis) complexed to DAB-Tf for 2h, before being washed and processed for fluorescence microscopy and flow cytometer analysis as described above.

#### 2.3.4. *In vitro* transfection

Transfection efficacy of the DNA carried by DAB-Tf dendrimer was assessed with a plasmid DNA encoding  $\beta$ -galactosidase (pCMV  $\beta\text{gal}$ ), using a  $\beta$ -galactosidase transfection assay. bEnd.3 cells were seeded at a density of 2 000 cells/well in 96-well plates ( $n=15$ ). After 72 h incubation, the cells were treated with the DAB-Tf dendriplex at the dendrimer: DNA weight ratio of 10:1, which has previously been shown to give the highest transfection on other cancer cell lines [14, 15]. DNA concentration (10  $\mu\text{g/mL}$ ) was kept constant for all the formulations tested. Naked DNA served as a negative control, DAB-DNA (dendrimer: DNA weight ratio 5:1) served as a positive control. After 72 h incubation, cells were lysed with 1X passive lysis buffer (PLB) (50  $\mu\text{L/well}$ ) for 20 min. The cell lysates were subsequently analyzed for  $\beta$ -galactosidase expression. Briefly, 50  $\mu\text{L}$  of the assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL o-nitrophenol- $\beta$ -galactopyranoside, 200 mM sodium phosphate buffer, pH 7.3) were added to each well containing the lysates. After 2 h incubation at 37°C, the absorbance of the samples was read at 405 nm with a Multiscan Ascent<sup>®</sup> plate reader (Thermo Scientific, Waltham, MA).

#### 2.4. *In vivo* study

##### 2.4.1. *Animals*

Female BALB/c mice were housed in groups of five at 19°C to 23°C with a 12-h light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, Grimston, UK) with mains water *ad libitum*. The *in vivo* experiments described below were approved by the local ethics committee and performed in accordance with the UK Home Office regulations.

##### 2.4.2. *Biodistribution of gene expression*

The biodistribution of gene expression was visualized by bioluminescence imaging, using an IVIS Spectrum<sup>®</sup> (PerkinElmer, Waltham, MA).

To determinate the treatment duration leading to the highest gene expression, female BALB/c mice ( $n= 3$ , initial mean weight: 20 g) were injected intravenously with a single dose of DAB-Tf carrying luciferase expression plasmid (50  $\mu\text{g}$  of DNA). They were then intraperitoneally injected with the luciferase substrate D-luciferin (150 mg/kg body weight) after various treatment durations and anaesthetized by isoflurane inhalation. Light emission was measured 10 min after injection of the D-luciferin solution, for 2 min, using Living Image<sup>®</sup> software (PerkinElmer, Waltham, MA). The resulting pseudo-color images represent the spatial distribution of photon counts within the animal. Identical illumination settings were used for acquiring all images [17].

A similar procedure was then performed at the optimum treatment duration to compare the distribution of gene expression resulting from the single intravenous injection of DAB-Tf and DAB dendriplexes encoding luciferase (50  $\mu\text{g}$  of DNA).

Biodistribution of gene expression was also quantified using a  $\beta$ -galactosidase reporter gene expression assay. Groups of mice ( $n= 5$ ) were injected intravenously with a single dose of DAB-Tf and DAB dendriplexes encoding luciferase (50  $\mu\text{g}$  of DNA). They were sacrificed 24 h after injection and their organs were removed, frozen in liquid nitrogen, before being analyzed for their  $\beta$ -galactosidase levels as previously described [18].

##### 2.4.3. *Distribution of gene expression within the brain*

Distribution of gene expression within the brain was qualitatively assessed by fluorescence microscopy imaging of the brain sections of mice treated with DAB-Tf dendriplex encoding

tdTomato. Mice were intravenously injected with a single dose of DNA encoding tdTomato, naked or complexed to DAB-Tf and DAB dendrimers (50 µg of DNA). They were sacrificed 24 h after injection and their brains were removed, fixed in a solution of 10% formalin for 48h. Following fixation, the brains were dehydrated through an ethanol gradient for 8h30, cleared in xylene for 2h30, before being embedded in paraffin wax. Coronal sections were cut at a thickness of 4 µm in different brain areas (anterior, median and posterior) and left in a 37°C oven overnight before being stained with hematoxylin and eosin (H&E) according to standard procedures. The brain sections were then examined using an E600FN Upright Epifluorescence microscope. Positivity for tdTomato expression in the brain was assessed at an excitation wavelength of 554 nm and emission wavelength of 581 nm.

### 2.5. Statistical Analysis

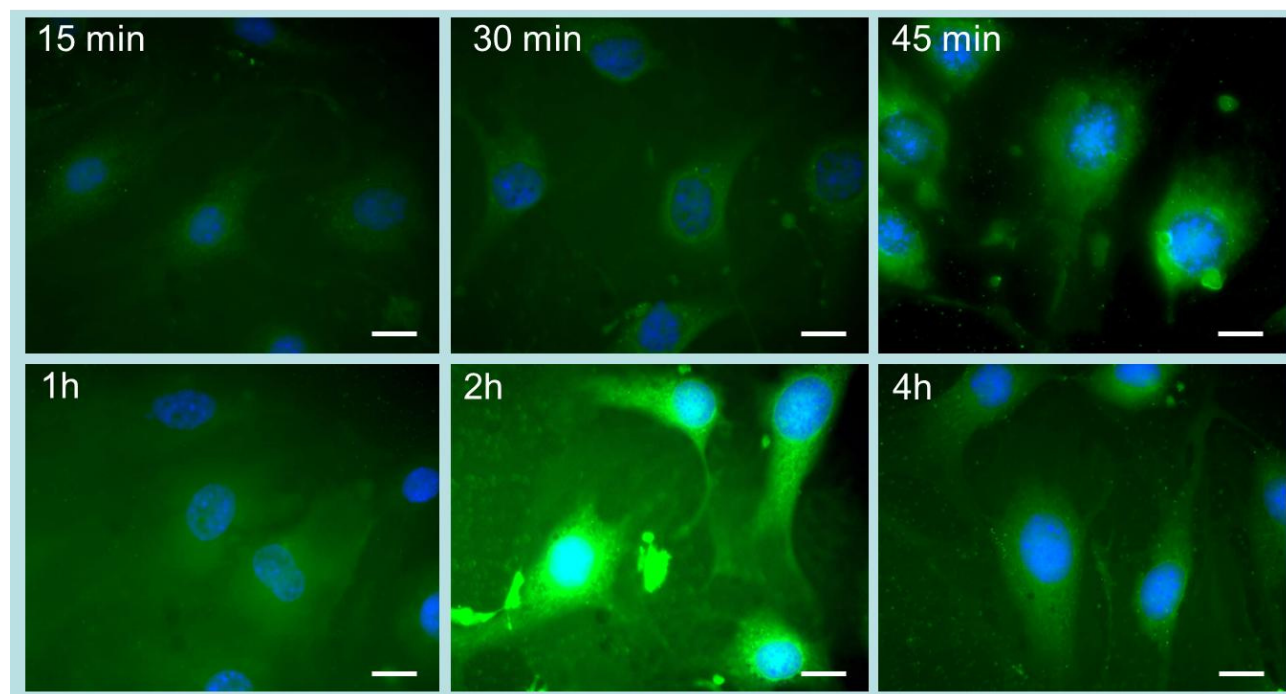
Results were expressed as means  $\pm$  standard error of the mean (S.E.M). Statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey multiple comparison post-test (Minitab<sup>®</sup> software, State College, PE). Differences were considered statistically significant for P values lower than 0.05.

## 3. Results and discussion

### 3.1. *In vitro* biological characterization

#### 3.1.1. Cellular uptake

The uptake of Cy3-labeled DNA complexed to DAB-Tf by bEnd.3 cells was qualitatively confirmed by epifluorescence microscopy. Cy3-labeled DNA was disseminated in the cytoplasm from as early as 15 min after the start of the treatment. Following various treatment durations, the DNA uptake in cells appeared to be most pronounced after treatment with DAB-Tf dendriplex incubated with the cells for 2 h (Fig. 1).

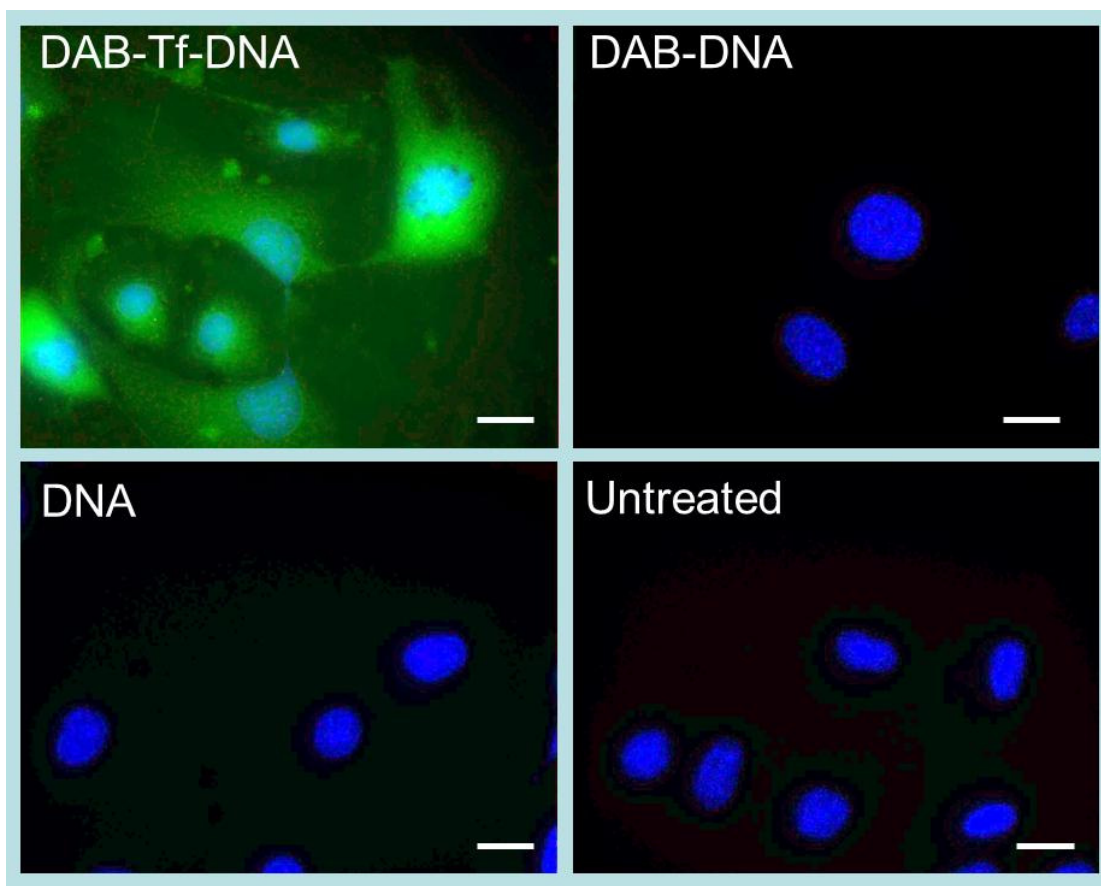


**Figure 1.** Epifluorescence microscopy imaging of the cellular uptake of Cy3- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, after incubation for 15 min, 30 min, 45 min, 1 h, 2 h or 4 h with bEnd.3 cells (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10 µm).

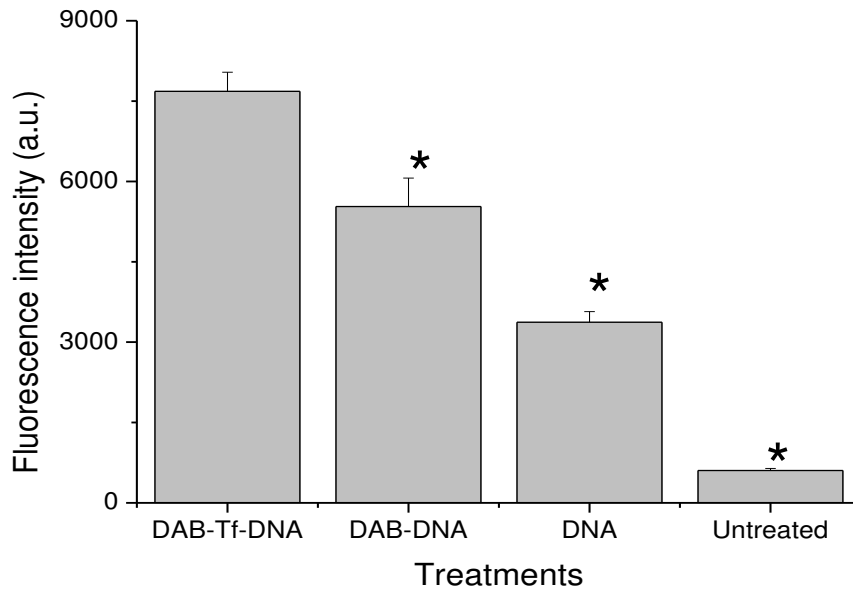


We then evaluated the cellular uptake of Cy3-labeled DNA either administered as complexed to DAB-Tf and DAB, or as a solution, following 2 h incubation (Fig. 2). The treatment of the cells with DAB-Tf dendriplex resulted in a pronounced DNA uptake in the cytoplasm of the cells. By contrast, cells treated with DAB dendriplex or DNA solution did not show any Cy3-derived fluorescence, highlighting the need of a targeted delivery system to carry DNA to bEnd.3 cells. The diffuse Cy3-derived fluorescence may be due to the homogeneous distribution of the fluorescently-labeled DNA in the cytoplasm following the escape of the DAB-Tf dendriplex from the endosomes and the release of the DNA from its dendritic carrier.

These results were quantitatively confirmed by flow cytometry (Fig. 3). Cellular fluorescence was highest following treatment with DAB-Tf dendriplex ( $7682 \pm 355$  arbitrary units (a.u.)). It was respectively about 1.4-fold and 2.3-fold higher than the cellular fluorescence observed following treatment with DAB dendriplex ( $5531 \pm 530$  a. u.) and DNA solution ( $3370 \pm 199$  a. u.).



**Figure 2.** Epifluorescence microscopy imaging of the cellular uptake of Cy3- labeled DNA (2.5  $\mu$ g/ well) either complexed with DAB-Tf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10  $\mu$ m).



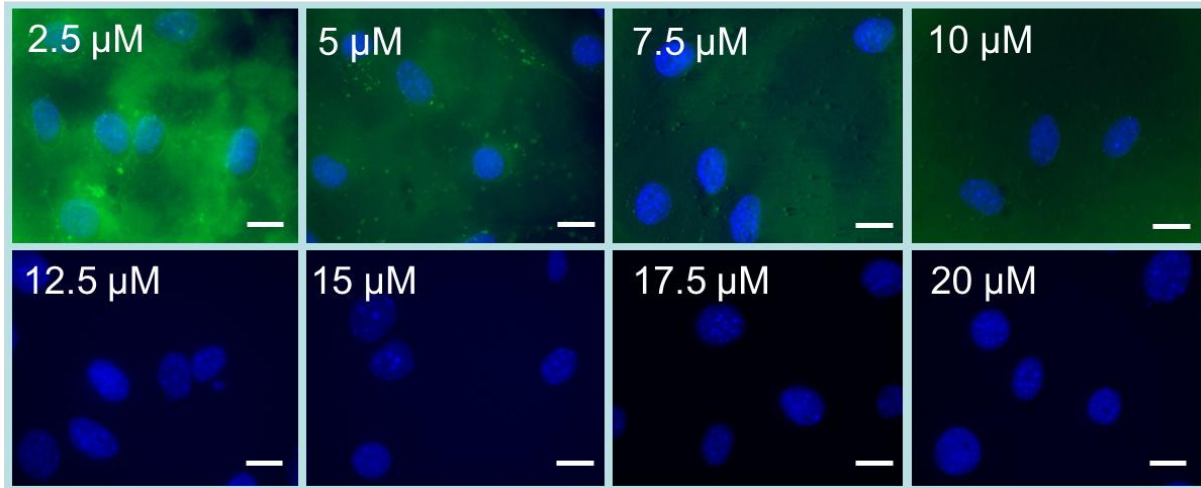
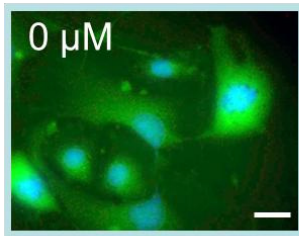
**Figure 3.** Flow cytometry quantification of the cellular uptake fluorescein- labeled DNA (2.5  $\mu\text{g}/\text{well}$ ) either complexed with DAB-Tf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (n=15) \* : P <0.05 compared with DAB-Tf-DNA.

In this study, we successfully demonstrated that the conjugation of Tf on DAB improved DNA uptake by bEnd.3 murine brain capillary endothelial cells, compared to control dendriplex and naked DNA treatments. Tf receptor-mediated uptake of DNA has been widely studied on cancer cell lines and brain capillary endothelial cells [11, 14, 15, 19-30]. Although the differences between treatments were less pronounced in our study, our results were in accordance with previous data obtained by Ko and colleagues [24], who revealed that the uptake of TfR-targeting biotinylated PEG-stabilized liposomes encapsulating PEI/ oligodeoxynucleotide was about 3-fold higher compared to that of non-targeted nanoparticles, in bEnd.5 mouse brain endothelial cell line. This outcome was also in line with our previous studies, in which we demonstrated that the conjugation of Tf to DAB increased the DNA uptake by T98G glioblastoma, PC-3M-luc-C6, DU145 and LNCaP prostate cells overexpressing Tf receptors [14, 19].

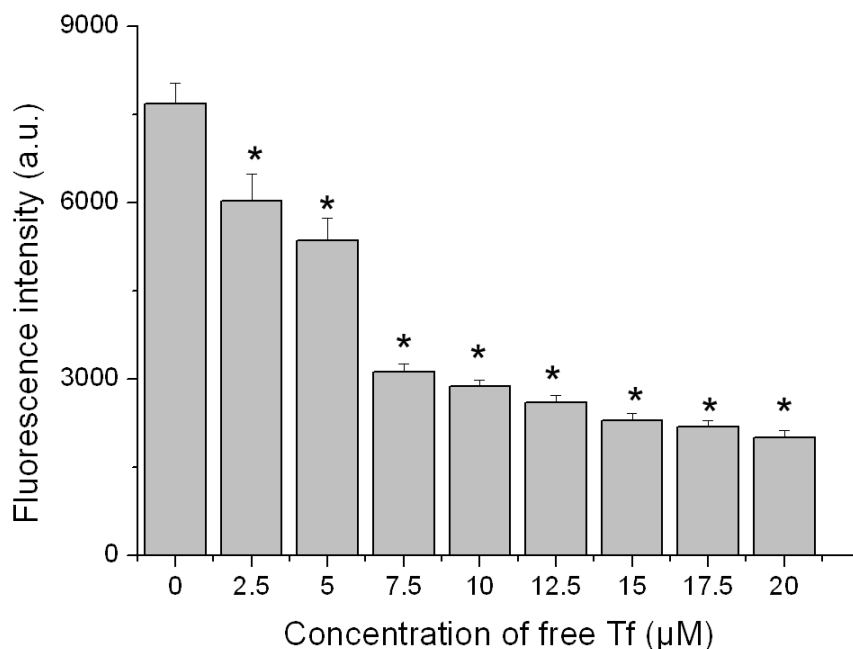
### 3.1.2. Mechanisms of cellular uptake of DNA complexed to DAB-Tf dendriplex

Pre-treatment of the bEnd.3 cells with various concentrations of free Tf significantly decreased the cellular uptake of fluorescein-labeled DNA complexed to DAB-Tf with increasing concentrations of free Tf, to reach a plateau at Tf concentrations higher than 12.5  $\mu\text{M}$  (Fig. 4 and 5). At a Tf concentration of 20  $\mu\text{M}$ , the cellular uptake of fluorescently-labeled DNA was 3.8-fold lower than that observed with DAB-Tf dendriplex without pre-Tf treatment (respectively  $2010 \pm 122$  a. u. and  $7682 \pm 355$  a. u.).





**Figure 4.** Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of Cy3-labeled DNA (2.5  $\mu\text{g}/\text{well}$ ) complexed with DAB-Tf, following pre-treatment with various concentrations of free Tf (ranging from 2.5  $\mu\text{M}$  to 20  $\mu\text{M}$ ). (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10  $\mu\text{m}$ ).



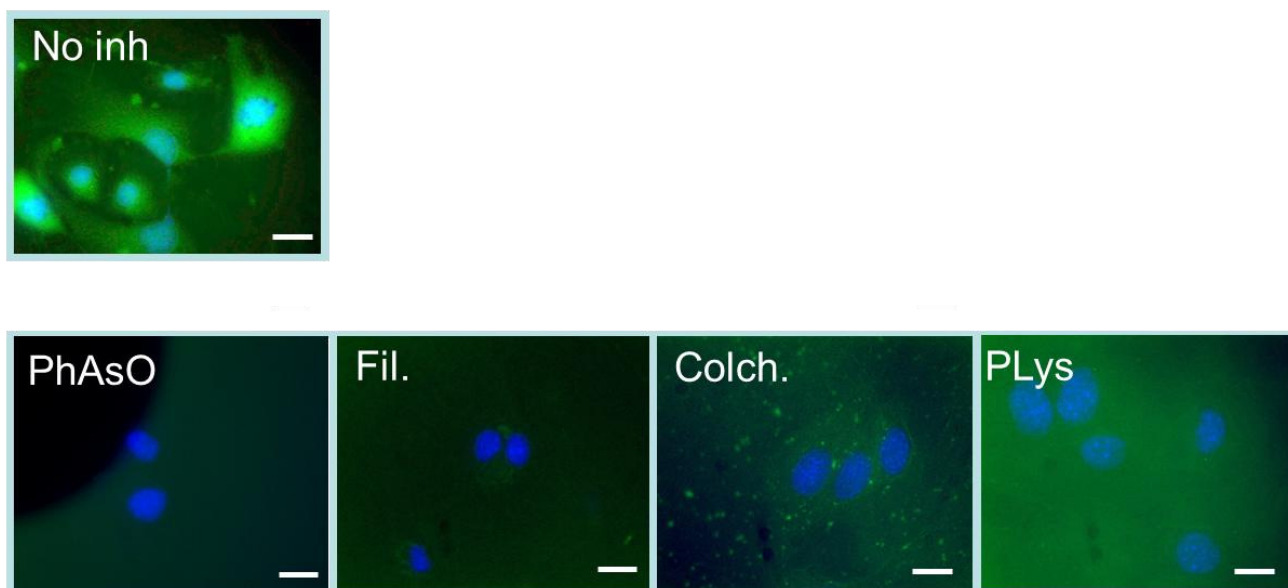
**Figure 5.** Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein-labeled DNA (2.5  $\mu\text{g}/\text{well}$ ) complexed with DAB-Tf, following pre-treatment with various concentrations of free Tf (ranging from 2.5  $\mu\text{M}$  to 20  $\mu\text{M}$ ) (n=15), \* : P < 0.05 compared with DAB-Tf-DNA.

The cellular uptake of fluorescein-labeled DNA complexed to DAB-Tf was also partially inhibited by phenylarsine oxide, filipin, colchicine and poly-L-lysine (Fig. 6 and 7). Colchicine and phenylarsine oxide caused the most significant inhibition, with a cellular uptake respectively decreased by 2.3-fold and 2.1-fold compared to that observed with DAB-Tf dendriplex without inhibitory treatment (respectively  $3316 \pm 251$  a. u. and  $3614 \pm 140$  a.u. following pre-treatment with colchicine and phenylarsine oxide). Filipin and poly-L-Lysine appear to be less effective inhibitors, leading to a cellular uptake decreased by respectively 1.7-fold and 1.3-fold compared to DAB-Tf dendriplex without pre-treatment (respectively  $4532 \pm 201$  a. u. and  $5974 \pm 192$  a.u. following pre-treatment with filipin and poly-L-Lysine).

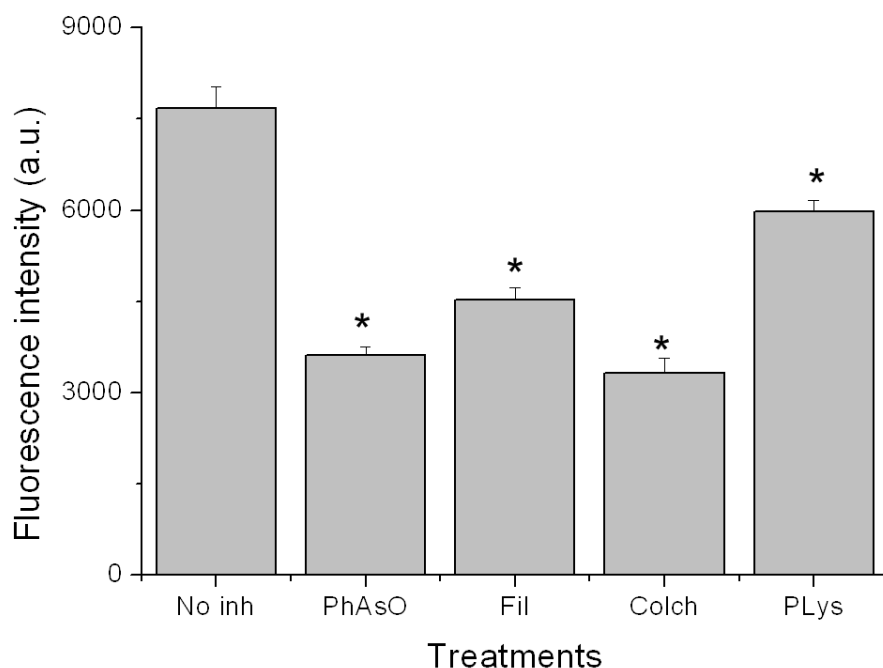
These inhibitors act on various endocytic mechanisms on the BBB. Phenylarsine oxide is an inhibitor of clathrin-mediated endocytosis (which is a requisite for receptor-mediated endocytosis) [31]. Filipin is known to block the caveolae-mediated process in non-specific adsorptive endocytosis [32]. Colchicine inhibits macropinocytosis [33], which provides non-specific endocytosis of macromolecules, whereas cationic poly-L-Lysine can inhibit the uptake of cationic delivery systems.

The cellular uptake of DNA complexed to DAB-Tf was therefore related to endocytosis processes, including clathrin-mediated endocytosis, macropinocytosis, and to a lesser extent caveolae-mediated endocytosis. The zeta potential of DAB-Tf dendriplex was slightly cationic (1.03 mV) [14], which limited the possible inhibitory role of poly-L-lysine. These results suggested that both receptor- and adsorptive-mediated mechanisms might contribute to the cellular uptake of DNA complexed to DAB-Tf.

Increasing amounts of Tf could significantly inhibit the cellular uptake of DNA complexed to DAB-Tf, suggesting that the Tf receptor-mediated mechanism might be the main mechanism of cellular internalization of DNA complexed to DAB-Tf.



**Figure 6.** Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of Cy3-labeled DNA ( $2.5 \mu\text{g}/\text{well}$ ) complexed with DAB-Tf, following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide (“PhAsO”), filipin (“Fil.”), colchicine (“Colch.”) and poly-L-lysine (“PLys”). (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10  $\mu\text{m}$ ).

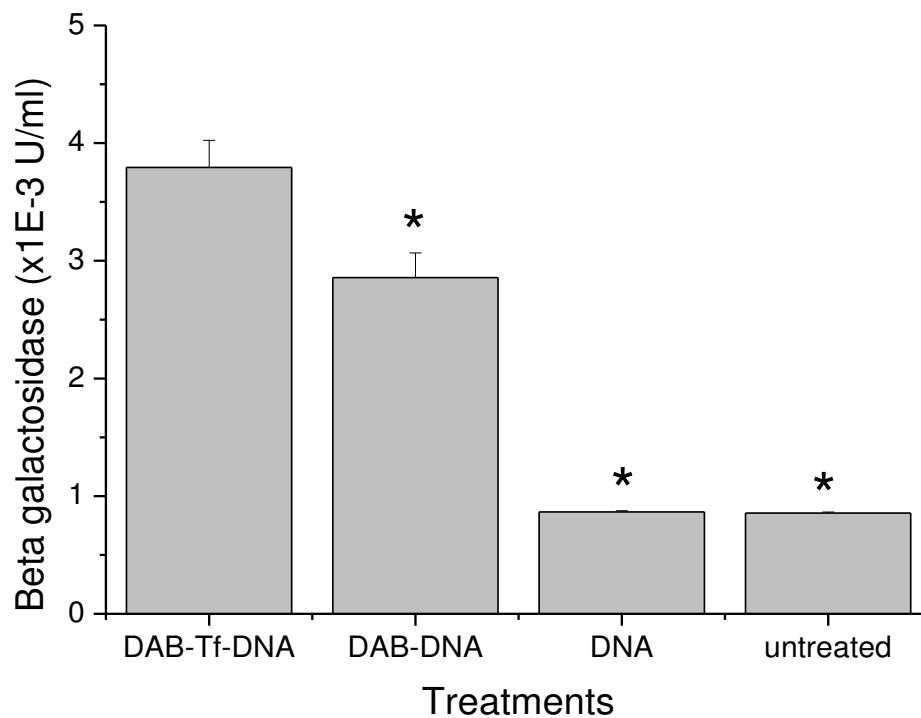


**Figure 7.** Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein-labeled DNA (2.5  $\mu$ g/ well) complexed with DAB-Tf, following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide (“PhAsO”), filipin (“Fil.”), colchicine (“Colch.”) and poly-L-lysine (“PLys”). (n=15), \* : P <0.05 compared with DAB-Tf-DNA.

### 3.1.3. *In vitro* transfection

The conjugation of Tf to DAB dendriplex led to an increased transfection compared to unconjugated DAB dendriplex on bEnd.3 cells (Fig. 8). Gene expression following treatment with DAB-Tf dendriplex was 1.3-fold higher than following treatment with DAB dendriplex ( $3.79 \times 10^{-3} \pm 0.23 \times 10^{-3}$  U/mL and  $2.85 \times 10^{-3} \pm 0.21 \times 10^{-3}$  U/mL respectively for DAB-Tf and DAB dendriplexes).

The treatment of bEnd.3 cells with Tf-bearing and DAB dendriplexes resulted in an increase in gene expression by about 4.3-fold and 3.3-fold respectively, compared to that observed following treatment with naked DNA ( $0.87 \times 10^{-3} \pm 0.01 \times 10^{-3}$  U/mL). The cells treated with naked DNA did not show any significant transfection increase compared to untreated cells.

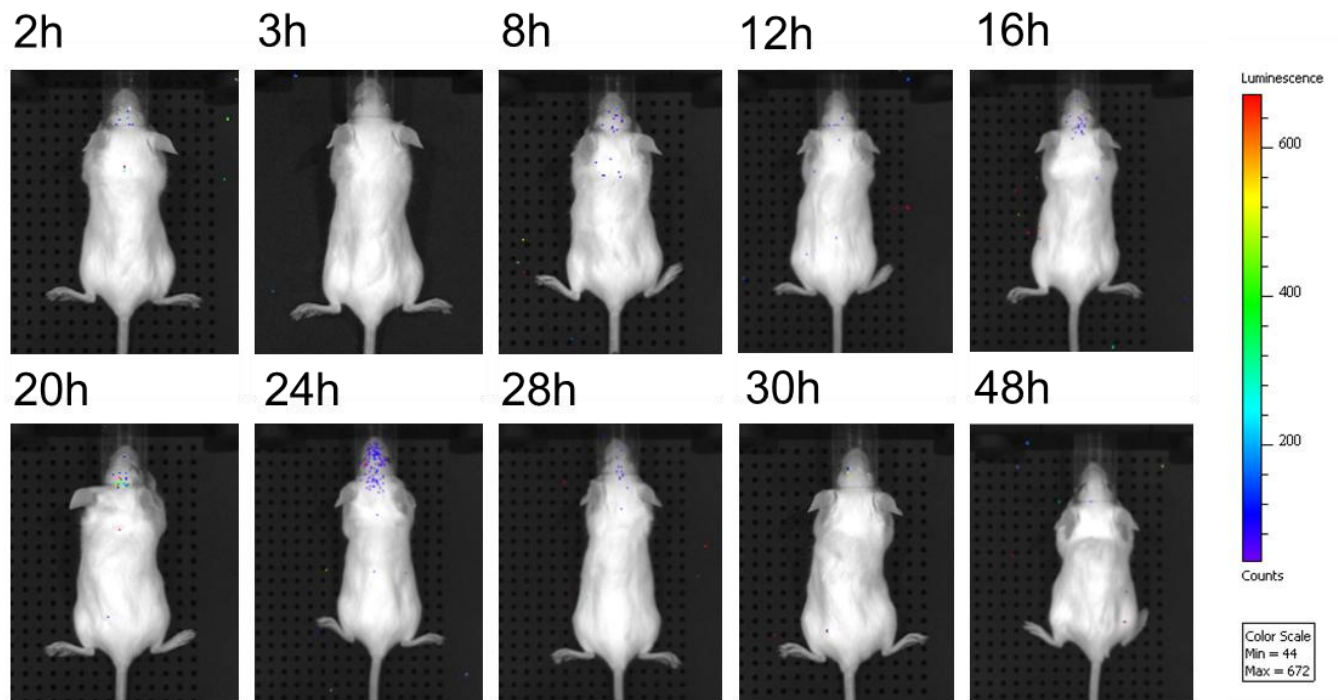


**Figure 8.** Transfection efficacy of DAB-Tf and DAB dendriplexes in bEnd.3 cells. DAB-Tf and DAB dendriplexes were dosed at their optimal dendrimer: DNA ratio of 10:1 and 5:1 respectively. Results are expressed as the mean  $\pm$  SEM of three replicates (n=15). \* : P < 0.05 compared with DAB-Tf-DNA.

The enhanced  $\beta$ -gal expression following DAB-Tf dendriplex treatment most likely resulted from the improved cellular uptake observed with this treatment. Both increases were of the same magnitude (1.4-fold for cellular uptake, 1.3-fold for gene expression compared to non-targeted DAB dendriplex treatment). These transfection results were in line with those described in the literature. For example, Huang and colleagues reported that the luciferase expression in brain capillary endothelial cells treated with Tf-bearing PEG-PAMAM dendriplex was 1.8-fold of that obtained with PAMAM dendriplex and PEG-PAMAM dendriplex [26]. In our previous experiments, the conjugation of Tf on DAB dendriplex already led to similar increases of gene expression, by 1.3-fold on both T98G and PC-3 cells, and by 2.2-fold on A431 cells, compared to that observed following treatment with DAB dendriplex [14, 19].

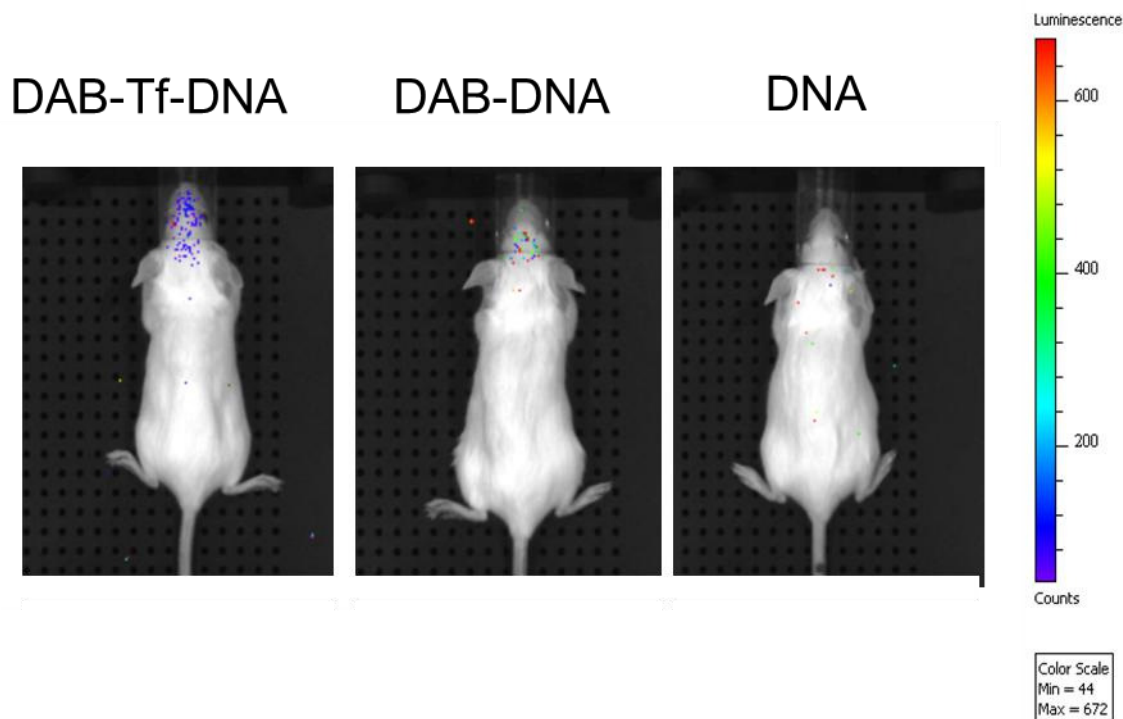
### 3.2. *In vivo* study

Distribution of gene expression following intravenous injection of DAB-Tf dendriplex encoding luciferase was first qualitatively assessed by luminescence imaging, at various treatment durations. Gene expression appeared to be mainly located in the brain of the mice. The highest gene expression level was found 24 h following injection of the treatment (Fig. 9).



**Figure 9.** Bioluminescence imaging of gene expression after intravenous administration of DAB-Tf dendriplex (50 µg DNA administered). The mice were imaged using the IVIS Spectrum at various durations after injection of the treatment. The scale indicates surface radiance (photons/s/cm<sup>2</sup>/steradian).

Gene expression following administration of DAB-Tf dendriplex was then compared to that observed following administration of DAB dendriplex and DNA only, 24 h after administration of the treatments. The level of gene expression in the brain appeared to be highest following treatment with DAB-Tf dendriplex (Fig. 10). In addition, luciferase expression appeared to be very limited in organs other than brain. This might be explained by the threshold of the technique that probably allowed only the most intensely luminescent tissues to be analyzed.



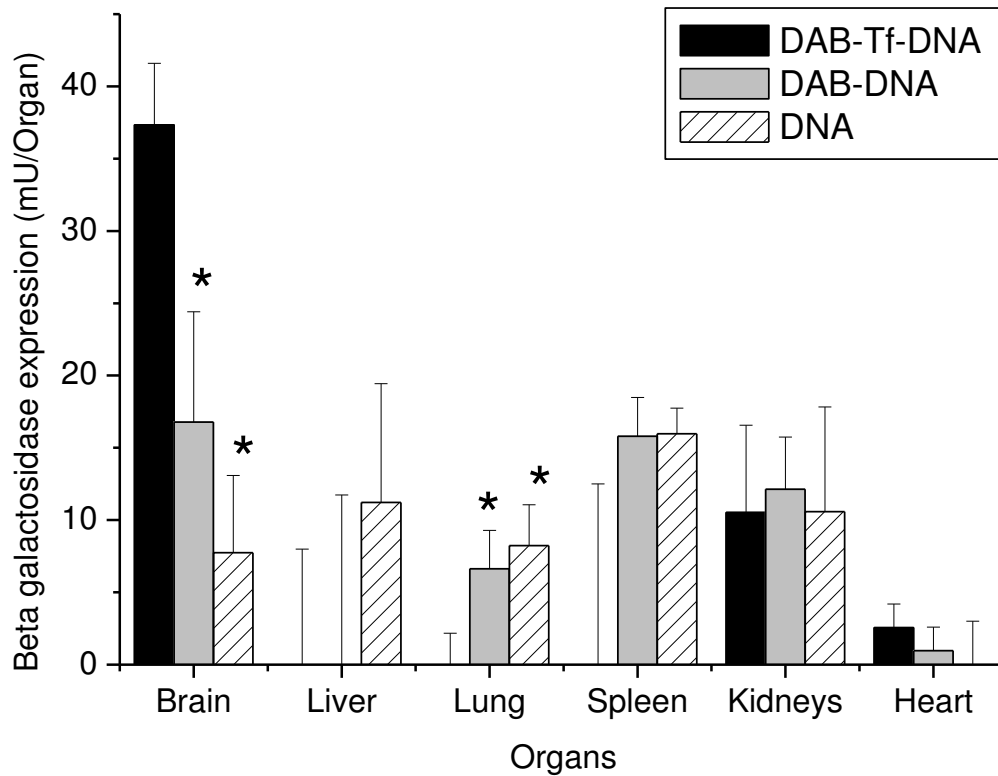
**Figure 10.** Bioluminescence imaging of gene expression after intravenous administration of DAB-Tf and DAB dendriplexes (50 µg DNA administered). (Controls: DNA solution, untreated cells). The mice were imaged using the IVIS Spectrum 24 h after injection of the treatments. The scale indicates surface radiance (photons/s/cm<sup>2</sup>/steradian).

These results were confirmed by quantification of gene expression in the major organs of the mice. The intravenous administration of DAB dendriplex led to gene expression mainly in the brain ( $16.7 \pm 7.6$  mU  $\beta$ -galactosidase per organ) and the spleen ( $15.8 \pm 2.7$  mU  $\beta$ -galactosidase per organ), followed by the kidneys ( $12.1 \pm 3.6$  mU  $\beta$ -galactosidase per organ) and then the lung ( $6.6 \pm 2.6$  mU  $\beta$ -galactosidase per organ) (Fig. 11).

By contrast, the conjugation of Tf to DAB significantly increased by more than 2-fold the gene expression in the brain ( $37.3 \pm 4.2$  mU  $\beta$ -galactosidase for DAB-Tf dendriplex), while there was very little  $\beta$ -galactosidase detected in the liver, the lung and the spleen. In the kidneys and the heart, gene expression reached similar levels to what was observed following treatment with DAB dendriplex ( $10.5 \pm 6.1$  mU and  $12.1 \pm 3.6$  mU  $\beta$ -galactosidase per organ in the kidneys for respectively DAB-Tf dendriplex and DAB dendriplex,  $2.5 \pm 1.6$  mU and  $0.9 \pm 1.6$  mU  $\beta$ -galactosidase in the heart for respectively DAB-Tf dendriplex and DAB dendriplex).  $\beta$ -galactosidase gene expression in the brain was at least 3-fold higher than in any peripheral organs tested in this study.

The amount of Tf injected as DAB-Tf was much higher than the endogenous amount of Tf in the plasma ( $2.68$  µg, corresponding to an endogenous plasma concentration of  $25$  µM [34], which limited the risk of competition for binding to the TfR.

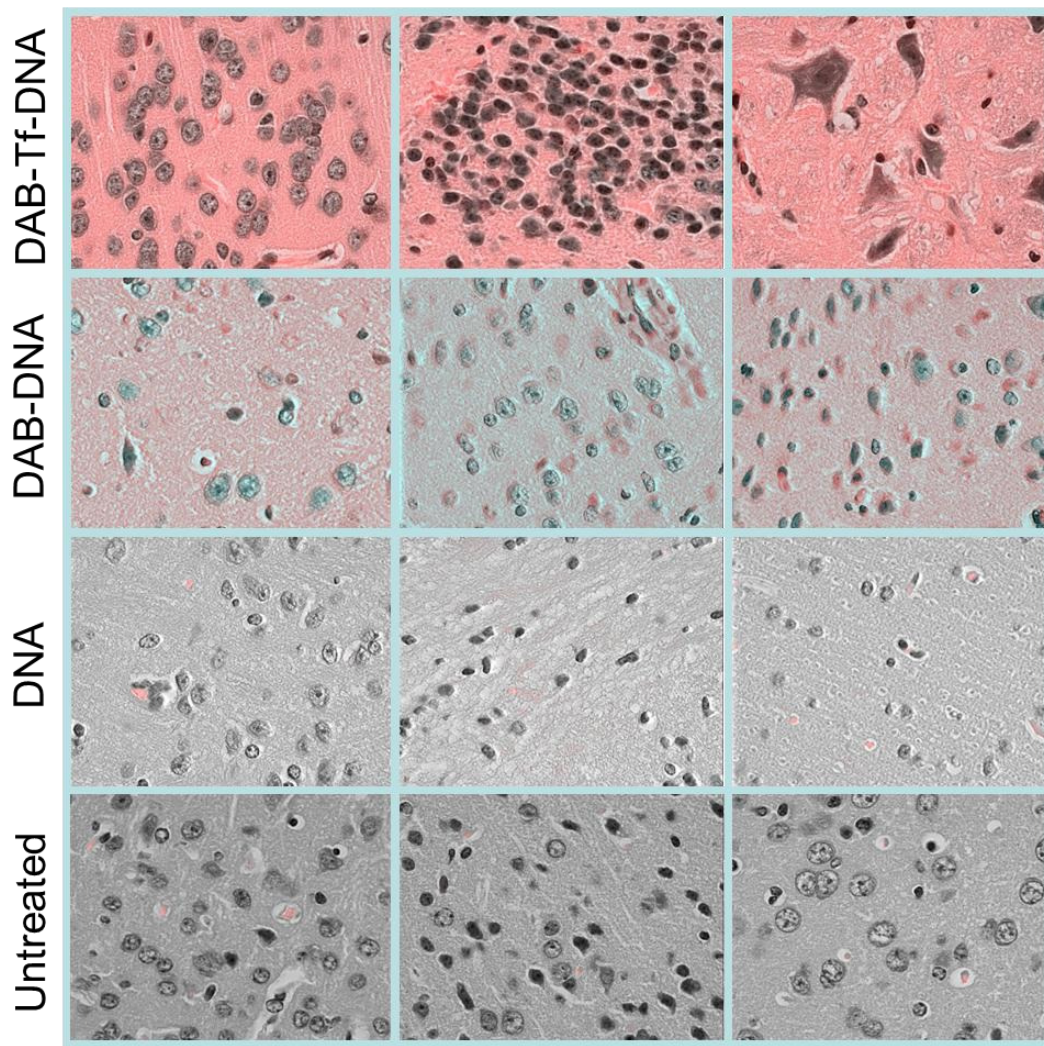
We have chosen to use a  $\beta$ -galactosidase expression assay for quantifying gene expression in the organs, as the spectrofluorimetric quantification of the reaction product 7-hydroxy-9H-(1, 3-dichloro-9, 9-dimethyl-acridin-2-one (DDAO) in the red part of the spectrum avoided interferences from hemoglobin which hamper many *in vivo* quantification assays [18, 35].



**Figure 11.** Biodistribution of gene expression after a single intravenous administration of DAB-Tf and DAB dendriplexes (50  $\mu$ g DNA administered). Results were expressed as milliunits  $\beta$ -galactosidase per organ (n=5). \*: P < 0.05 compared with DAB-Tf-DNA for each organ.

Within the brain, gene expression was the highest following administration of DAB-Tf dendriplex (Fig. 12). It was homogenously distributed in the brain parenchyma in all the sections of the brain we observed, but did not appear to have reached the neurons and glial cells. By contrast, tdTomato gene expression was very limited in the brain following administration of DAB dendriplex. Some autofluorescence artefacts were visible in the brain treated with naked DNA or left untreated.





**Figure 12.** Epifluorescence microscopy imaging of the distribution of gene expression within the brain after a single intravenous injection of tdTomato- encoded DNA (50  $\mu$ g) either complexed with DAB-Tf, DAB or in solution (Magnification: x 60).

This communication presents evidence that DAB-Tf dendriplex led to improved gene expression in the brain following intravenous administration. To our knowledge, it is the first time that the intravenous administration of a Tf-bearing non-viral delivery system resulted in such intense effects.

Other groups have already demonstrated gene transfer capabilities of Tf-bearing and TfR-targeting gene delivery systems following intravenous administration, but with much smaller gene expression in the brain and much larger non-specific expression in other organs. For example, the intravenous administration of liposomes encapsulating polyethylenimine/  $^{32}$ P- oligodeoxynucleotide polyplexes and conjugated to 8D3 anti-mouse Tf receptor monoclonal antibody, resulted in an increased brain uptake, but with the tracer activity being found mainly in liver, spleen and kidneys [24].

Similar gene expression levels and tissue expression patterns were seen when lipoplexes encoding luciferase were conjugated with the OX26 anti-rat Tf receptor monoclonal antibody. Following 48 h after intravenous administration of this lipoplex, the exogenous gene was expressed in brain with levels as high as 0.2 pg/mg protein, but was also mainly

found in peripheral tissues such as liver, spleen and lung [36]. Another study using a similar lipoplex reported that the luciferase gene expression in brain was comparable to that of lung or spleen [22]. Similarly, the injection of 8D3-bearing PEGylated immunolipoplexes encoding  $\beta$ -galactosidase also led to a similar pattern of distribution, with gene expression being found in the brain, but mostly in liver and spleen [20, 22].

The differences in the biodistribution of gene expression may be explained by the targeting of different TfR and the use of Tf instead of anti-TfR antibodies as a targeting moiety. Both TfR1 and TfR2 are members of the Tf receptor family. TfR1 is expressed at low levels in most human tissues, but is highly expressed on the vascular endothelium of brain capillaries [7]. It is also expressed at levels up to 100-fold higher than those on normal cells on highly proliferative cells such as cancer cells [37], making this receptor a promising target for the delivery of therapeutics to the brain and cancer cells. By contrast, the  $\alpha$ -transcript product of TfR2 is mostly expressed on hepatocytes, while its  $\beta$ -transcript is present on a wide range of tissues but at very low levels [38]. In addition, TfR1 has a much higher affinity for Tf than TfR2 (25-fold higher) [38], which is not the case for anti-TfR monoclonal antibodies. TfR1 would therefore be a more efficient target for transferrin-mediated gene delivery to the brain or cancer cells.

Previous studies using the dendrimer polyamidoamine (PAMAM) instead of DAB have shown that the gene expression of intravenously administered Tf-bearing PAMAM dendriplex was about 2-fold higher in the brain than that of non-targeted dendriplex. Gene expression of luciferase, however, was mainly found in heart and lung [26].

In a previous study done on tumor-bearing mice, we demonstrated that the intravenous administration of DAB dendrimer conjugated to transferrin (Tf), whose receptors are also overexpressed on cancer cells, resulted in gene expression mainly in the tumors after intravenous administration [14]. Thus, DAB-Tf dendrimer complexed to a TNF $\alpha$ -encoding DNA led to a rapid and sustained tumor regression over one month, resulting in complete suppression of 90% of the tested A431 tumors and regression of the remaining 10% [14]. In this study, we wanted to investigate if the targeting properties of this dendrimer could lead to an enhanced delivery of DNA to the brain after intravenous administration, on mice without tumors. We demonstrated that this is indeed the case.

In conclusion, we have demonstrated that transferrin-bearing DAB polypropylenimine dendrimer led to an increased gene expression in the brain, which was at least 3-fold higher than in any tested peripheral organs. Transferrin-bearing DAB dendrimer is therefore a highly promising delivery system for gene delivery to the brain and will be further investigated to optimize its therapeutic potential.

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## FIGURE CAPTIONS

**Figure 1.** Epifluorescence microscopy imaging of the cellular uptake of Cy3- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, after incubation for 15 min, 30 min, 45 min, 1 h, 2 h or 4 h with bEnd.3 cells (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10 µm).

**Figure 2.** Epifluorescence microscopy imaging of the cellular uptake of Cy3- labeled DNA (2.5 µg/ well) either complexed with DAB-Tf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10 µm).

**Figure 3.** Flow cytometry quantification of the cellular uptake of fluorescein- labeled DNA (2.5 µg/ well) either complexed with DAB-Tf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (n=15) \* : P <0.05 compared with DAB-Tf-DNA.

**Figure 4.** Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of Cy3- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, following pre-treatment with various concentrations of free Tf (ranging from 2.5 µM to 20 µM). (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10 µm).

**Figure 5.** Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, following pre-treatment with various concentrations of free Tf (ranging from 2.5 µM to 20 µM) (n=15), \* : P <0.05 compared with DAB-Tf-DNA.

**Figure 6.** Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of Cy3- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys"). (Blue: nuclei stained with DAPI (excitation: 405 nm, laser line bandwidth: 415-491 nm), green: Cy3-labeled DNA (excitation: 543 nm, laser line bandwidth: 550-620 nm) (Bar: 10 µm).

**Figure 7.** Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein- labeled DNA (2.5 µg/ well) complexed with DAB-Tf, following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys"). (n=15), \* : P <0.05 compared with DAB-Tf-DNA.

**Figure 8.** Transfection efficacy of DAB-Tf and DAB dendriplexes in bEnd.3 cells. DAB-Tf and DAB dendriplexes were dosed at their optimal dendrimer: DNA ratio of 10:1 and 5:1 respectively. Results are expressed as the mean ± SEM of three replicates (n=15). \* : P <0.05 compared with DAB-Tf-DNA.

**Figure 9.** Bioluminescence imaging of gene expression after intravenous administration of DAB-Tf dendriplex (50 µg DNA administered). The mice were imaged using the IVIS Spectrum at various durations after injection of the treatment. The scale indicates surface radiance (photons/s/cm<sup>2</sup>/steradian).

**Figure 10.** Bioluminescence imaging of gene expression after intravenous administration of DAB-Tf and DAB dendriplexes (50 µg DNA administered). (Controls: DNA solution, untreated cells). The mice were imaged using the IVIS Spectrum 24 h after injection of the treatments. The scale indicates surface radiance (photons/s/cm<sup>2</sup>/steradian).

**Figure 11.** Biodistribution of gene expression after a single intravenous administration of DAB-Tf and DAB dendriplexes (50 µg DNA administered). Results were expressed as milliunits β-galactosidase per organ (n=5). \* : P <0.05 compared with DAB-Tf-DNA for each organ.

**Figure 12.** Epifluorescence microscopy imaging of the distribution of gene expression within the brain after a single intravenous injection of tdTomato- encoded DNA (50 µg) either complexed with DAB-Tf, DAB or in solution (Magnification: x60).